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(54)【発明の名称】　蛋白質分離精製方法

(57)【要約】
【構成】　平均孔径が５０～１００ｎｍの親水性フィルターを用いることを特徴とする細胞混入溶液からの蛋白質分離精製方法。
【効果】　本発明の方法を行なえば、細胞培養からの有用蛋白質の分離精製工程において、工程初期の粗分離における精製度を著しく向上させ、蛋白回収率の向上、操作の簡略化、装置の省略等により製造コストの低減をもたらすことができる。

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【特許請求の範囲】
【請求項１】　平均孔径が５０～１００ｎｍの親水性フィルターを用いて、細胞混入溶液から蛋白質を分離精製することを特徴とする蛋白質分離精製方法。
【請求項２】　細胞混入溶液が細胞培養液であることを特徴とする請求項１記載の蛋白質分離精製方法。
【発明の詳細な説明】
【０００１】
【産業上の利用分野】本発明は、細胞混入溶液から蛋白質を効率よく分離精製する方法に関する。
【０００２】
【従来の技術】遺伝子操作や細胞融合等を利用して生産される細胞培養医薬品などのバイオ生産品は、その製造過程で目的とする生産物と細胞及び細胞片等の不純物とを分離精製することが必要であり、この分離精製工程がバイオ生産プロセスの心臓部の一つにあたる機能を果すことも周知である。
【０００３】従来、バイオ医薬品製造過程初期に細胞及び細胞片と目的蛋白質を分離精製する方法としては、まず、大まかな沈降・遠心分離等の操作を行い、次いで分離精製される上清液の中に不純物として残存する細かな細胞断片を分離することが通常行われてきた。その際、目的蛋白質を分離精製する方法としては、遠心分離、ゲル濾過、及び膜分離等の方法がとられてきた。
【０００４】しかし、本発明者等の検討によって、従来のこれら蛋白質分離精製方法にはいずれも未解決の問題があることが判ってきた。即ち、例えば遠心濾過法においては、装置が大がかりで装置の操作性が煩雑であるばかりか、どうしても遠心後の残渣に蛋白質が残り、最終蛋白質の回収率が低下してしまうという重大な欠点がある。
【０００５】また、ゲル濾過法は、遠心濾過法同様装置が大きく操作性が煩雑である上、運転コストがかかるといった欠点を有する。一方、膜による分離方法は、装置もコンパクトで、操作自体も簡単といった優れた特徴があるものの、従来使用されてきた除菌用フィルターでは、例えば０．２μｍ前後の微小細胞片の除去が不充分でかつすぐに目詰まりしてしまうため、目的蛋白質の分離精製を充分に行うに到らず実用的でないといった欠点があった。
【０００６】さらに、これらの従来知られているいずれの方法によっても、細胞片と目的蛋白質との分離精製度が不十分であり、そのためにバイオ生産品製造の最終工程で通常使われるクロマト精製工程の機能を低下させてしまうといった未解決問題もかかえていた。
【０００７】
【発明が解決しようとする課題】本発明の課題は、かかる上記の欠点を解消し、バイオ生産品製造工程初期での細胞特に細胞断片と目的蛋白質の分離精製を、優れた分離性能で行いかつ簡単に行なうことができる蛋白質分離

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精製方法を提供することである。
【０００８】
【課題を解決するための手段】本発明者らは、上記課題を達成すべく種々の細胞混入溶液の実状の解析から検討を開始し、その結果、本発明に到達するに致ったのである。すなわち、本発明は、平均孔径が５０～１００ｎｍの親水性フィルターを用いて細胞混入溶液から蛋白質を分離精製することを特徴とする細胞混入溶液からの蛋白質分離精製方法である。
【０００９】また、本発明は、この細胞溶液が細胞培養液であることを特徴とする蛋白質分離精製方法である。本発明におけるフィルターは、親水性フィルターである。親水性でないフィルターには蛋白質が吸着しやすく、蛋白回収率が低下する。更にフィルターが目詰まりして処理量が低下するため望ましくない。
【００１０】本発明におけるフィルターは、親水性素材であればどのようなものでも用い得るがセルロース系素材が好ましく、再生セルロース系素材からなるものが蛋白質の吸着が際立って少ないため特に好ましい。例えば特開昭６１－２５４２０２号公報、特開昭６１－２７４７０７号公報に記載の再生セルロース膜などが取り扱いも簡単であり好ましく使用できる。
【００１１】本発明におけるフィルターの平均孔径は５０～１００ｎｍでなくてはならない。５０ｎｍ以下であると、細胞片などの不純物による目詰まりの進行がすすみ、実用的でない。また、平均孔径が１００ｎｍ以上であると撹拌などによって生じる細胞片及びその他不純物の除去が不完全であり、蛋白質の精製度が低下する傾向が特に激しくなるため好ましくない。
【００１２】これらの事実は、本発明者らがフィルターの平均孔径を高度に制御する技術を駆使し、かつ細胞混入溶液を用いた多くの分離テストを行った結果初めて見出し得たものである。さらに、蛋白質の精製度、分離効率、濾過速度、濾過量などを総合的に評価すると、平均孔径が６０～８０ｎｍである方がより好ましい。
【００１３】本発明におけるフィルターの平均孔径とは、例えば特開昭６１－２５４２０２号公報、特開昭６１－２７４７０７号公報に記載されている平均孔径であり、下記式により算出されるものである。

$$2r_f = 2.0\,(J_v \cdot d \cdot \eta / \Delta P \cdot A \cdot Pr\rho)^{1/2}$$

$2r_f$：平均孔径　　J_v：濾過速度（ｍｌ／分）
ΔＰ：膜間差圧
Ａ：有効膜面積　　ｄ：膜厚（μｍ）　η：純水の粘度（センチポイズ）
$Pr\rho$：空孔率　である。
【００１４】本発明は、細胞及び細胞断片混入溶液から目的蛋白質を高精度・高効率で分離精製する発明であるが、バイオ生産物の製造工程で生じる細胞培養液から細胞及び細胞断片と蛋白質とを分離精製するのに特に適し

ている。本発明で用いるフィルターは、従来の濾過方法・装置に比較して、より簡単な濾過装置とすることができるし、また装置の操作もより簡単にすることができる。また本発明により、蛋白質の分離精製度及び蛋白質回収効率が極めて向上するため分離精製工程以降の後工程における精製工程を省略し得るし、更にクロマトの寿命が長くなるために精製コストを大巾に減らすことを期待できる。また、本発明は、ウィルスバリデーションされたフィルター装置を使用すれば、ＨＩＶ等のレトロウィルスなどの大きなウィルスの除去を同時に行なえるといったすぐれた特徴をも有する。

【００１５】

【実施例１】特開昭６１－２５４２０２号公報、特開昭６１－２７４７０７号公報記載の方法に準じ、セルロース濃度５．８％、アンモニア濃度４．７％の銅アンモニア溶液を調整し紡糸原液とした。この紡糸原液を２５±０．４℃に制御しつつ、環状紡口出口の外側（外径２．２ｍｍφ、内径１．６ｍｍφ）より、吐出量２．０ｍｌ／分で吐出させた。

【００１６】一方、アセトン濃度５３％、アンモニア濃度０．７％の水溶液を２５±０．４℃に制御して環状紡口出口の内側（０．４ｍｍφ）より０．８ｍｌ／分で吐出させた。吐出した糸状物を約１０ｍｍの距離を空中走行後、２５±０．４℃に制御したアセトン濃度３５％、アンモニア濃度０．２％の水溶液に導き、外溶液中で、平均孔径が７５ｎｍになるように制御しつつ５ｍ／分の速度で巻き取った。

【００１７】その後、固定状態で３％の硫酸溶液を使用して収縮、再生させ、次いで水洗した。得られた中空糸を、中空糸中の水をメタノールで置換後、２０℃で真空乾燥した。かくして得られた中空糸の外径は、３４５μｍ、膜厚は、３０μｍであった。平均孔径を測定したところ、７４±４ｎｍであった。この中空糸を使用し、公＊

＊知の方法で、膜面積０．０１ｍ² になるようにフィルター装置を組み立てた。

【００１８】一方、大腸菌を生理食塩水溶液に入れ、ホモジナイザーで撹拌・粉砕した。この撹拌液を電子顕微鏡写真で観察したところ０．１５～１００μｍの細胞片が観察された。この溶液に、この溶液１，０００ｍｌ当りウシ血漿由来のγ－グロブリン乾燥粉末を１．０ｇを溶解させた。得られた蛋白質溶液を当該フィルター装置で初期圧０．７ｋｇ／ｃｍ² で濾過した。濾過は、濾液総量が１リットルになるまで続けたが、濾過圧の上昇はほとんど認められず、このときの濾過圧は、０．７～０．８ｋｇ／ｃｍ² であった。濾液を電子顕微鏡で観察したところ、細胞片は認められなかった。また、濾過前の液と濾過後５０ｍｌ～１００ｍｌの液を分光光度計（波長２８０ｎｍ）で測定し、蛋白の透過性を測定した。結果を表１に示す。表１に示すとおり蛋白の透過率は１００％であった。

【００１９】なおこの蛋白透過率は、細胞片混入溶液からの蛋白質回収率に相当することは明らかである。

【００２０】

【比較例】フィルター装置が、当該フィルター装置の代わりに、ミリポア社製孔径０．２２μｍの除菌用フィルターを使う以外は、実施例１と同様の条件・方法で濾過及び測定を行なった。結果を表１にあわせて示す。表１に示すとおり、濾液量が１００ｍｌになった時点で濾過圧が著しく上昇し濾過が不可能となったため濾過を中止した。

【００２１】この濾液量を実施例のそれと比較すると、実施例１の濾液量のわずか１／１０に過ぎなかった。また、蛋白透過率も９０％と低く、濾液の電子顕微鏡写真には、細胞片が若干観察された。

【００２２】

【表１】

	濾液量	濾液中の細胞片の有無	蛋白透過率
実施例	1,000ml	な　し	100%
比較例	100ml	若干有り	90%

【００２３】

【発明の効果】本発明によれば、以上詳述のように、細胞混入溶液から目的蛋白質を高精度、高効率でかつ簡単に分離精製することができる。

PATENT ABSTRACTS OF JAPAN

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(54) METHOD FOR SEPARATING AND PURIFYING PROTEIN

(57)Abstract:
PURPOSE: To separate and purify a protein from a cell mixed solution in high precision and in high efficiency by using a hydrophilic filter having a given average particle diameter.
CONSTITUTION: A protein is separated from a cell mixed solution such as a cell culture solution by using a hydrophilic filter having 50-100nm average micropore diameter composed of a regenerated cellulose-based material.

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CLAIMS

[Claim(s)]
[Claim 1] The protein separation purification approach characterized by an average aperture carrying out separation purification of the protein from a cell mixing solution using the hydrophilic filter which is 50-100nm.
[Claim 2] The protein separation purification approach according to claim 1 characterized by a cell mixing solution being cell culture liquid.

[Translation done.]

DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[Industrial Application] This invention relates to the approach of carrying out separation purification of the protein efficiently from a cell mixing solution.
[0002]
[Description of the Prior Art] Biotechnology production articles, such as cell culture drugs produced using genetic manipulation, cell fusion, etc., need to carry out separation purification of the impurities, such as a product made into the purpose, a cell, and a piece of a cell, in that manufacture process, and it is also common knowledge to achieve the function in which this separation purification process hits one of the cores of a biotechnology production process.
[0003] Conventionally, as an approach of carrying out separation purification of a cell and the piece of a cell, and the purpose protein in early stages of a biomedicine manufacture process, rough sedimentation, centrifugal separation, etc. were operated first, and separating the fine cell fragment which remains as an impurity into the digestive liquor by which separation purification is subsequently carried out has usually been performed. As an approach of carrying out separation purification of the purpose protein, approaches, such as centrifugal separation, gel filtration, and membrane separation, have been taken in that case.
[0004] However, this invention person's etc. examination has shown that there are all unsolved problems in these conventional protein separation purification approach. That is, in a centrifugal filtration method, equipment is large-scale, and there is the operability of equipment being complicated and a serious fault that protein will surely remain in the residue after centrifugal, and the recovery of the last protein will fall, for example.
[0005] Moreover, gel filtration technique has the fault that operation cost starts, like a centrifugal filtration method the top where equipment is large and operability is complicated. on the other hand , although the separation approach of equipment by the film be compact and had the outstanding description that the actuation itself be easy , in order removal of the piece of a minute cell around 0.2 micrometers be inadequate , for example and to carry out blinding immediately with the filter for disinfection use conventionally , it had the fault that do not fully come to perform separation purification of the purpose protein , and it be practical .
[0006] Furthermore, by any approach learned conventionally [these], whenever [separation purification-with piece of cell and purpose protein] is inadequate, therefore it also had the open question of reducing the function of the chromatography purification process usually used by the final process of biotechnology production article manufacture.
[0007]
[Problem(s) to be Solved by the Invention] The technical problem of this invention is offering the protein separation purification approach which can be performed easily [cancel this above-mentioned fault, and perform the cell in the early stages of a biotechnology production article production process, especially separation purification of a cell fragment and the purpose protein by the outstanding separability ability, and].
[0008]
[Means for Solving the Problem] this invention persons start examination from the analysis of the actual condition of various cell mixing solutions that the above-mentioned technical problem should be attained, consequently are that of ****** reaching this invention. That is, this invention is the protein separation purification approach from the cell mixing solution characterized by an average aperture carrying out separation purification of the protein from a cell mixing solution using the hydrophilic filter which is 50-100nm.
[0009] Moreover, this invention is the protein separation purification approach characterized by this cell solution being cell culture liquid. The filter in this invention is a hydrophilic filter. Protein tends to stick to the filter which is not a hydrophilic property, and protein recovery falls. Furthermore, since a filter carries out blinding and throughput falls, it is not desirable.
[0010] If it is a hydrophilic material, anythings can be used, but the filter in this invention has a desirable cellulose system material, and especially since it is few, it is [the adsorption whose thing which consists of a regenerated-cellulose system material is protein is conspicuous, and] desirable. For example, the regenerated-cellulose film of a publication etc. can also use handling for JP,61-254202,A and JP,61-274707,A simply and preferably.
[0011] The average aperture of the filter in this invention must be 50-100nm. Advance of the blinding according that it is 50nm or less to impurities, such as a piece of a cell, progresses, and it is not practical. Moreover, in addition to this, removal of an impurity is imperfect, and since the piece of a cell produced by churning etc. as an average

aperture is 100nm or more, and the inclination for whenever [protein purification-] to fall become intense especially, it is not desirable.
[0012] These facts can be found out for the first time, as a result of making full use of the technique in which this invention persons control the average aperture of a filter to altitude and performing many separation tests using a cell mixing solution. Furthermore, it is more more desirable for an average aperture to be 60-80nm, if separation efficiency, filtration velocity, the amount of filtration, etc. are evaluated synthetically whenever [protein purification-].
[0013] The average aperture of the filter in this invention is an average aperture indicated by JP,61-254202,A and JP,61-274707,A, and it is computed by the following type.
2rf =2.0(Jv and d-eta/delta P-A-Prrho) 1/22rf : Average aperture Jv : Filtration velocity (a part for ml/) Differential pressure A between deltaP:film: Effective film surface product d: Thickness (micrometer) eta: Viscosity of pure water (centipoise)
Prrho: -- void content it is .
[0014] Although this invention is invention which is highly precise and efficient and carries out separation purification of the purpose protein from a cell and a cell fragment mixing solution, it is suitable for especially carrying out separation purification of a cell and a cell fragment, and the protein from the cell culture liquid produced in the production process of a biotechnology product. As compared with the conventional filtration approach and equipment, the filter used by this invention can be used as a easier filter, and can also simplify actuation of equipment more. Moreover, since the purification process in the back process after a separation purification process can be skipped by this invention since whenever [protein separation purification-], and protein recovery effectiveness improve extremely, and the life of chromatography becomes long further, it is expectable to reduce purification cost sharply. Moreover, this invention also has the outstanding description that big viruses, such as retroviruses, such as HIV, are removable to coincidence, if the filter equipment by which virus validation was carried out is used.
[0015]
[Example 1] According to the approach JP,61-254202,A and given in JP,61-274707,A, the cupro ammonium solution of 5.8% of cellulose concentration and 4.7% of ammonia concentration was adjusted, and it considered as the spinning undiluted solution. It was made to breathe out by part for 2.0ml/of discharge quantity from the outside (outer-diameter 2.2mmphi, bore 1.6mmphi) of an annular spinning port outlet, controlling this spinning undiluted solution at 25**0.4 degrees C.
[0016] On the other hand, the water solution of 53% of acetone concentration and 0.7% of ammonia concentration was controlled at 25**0.4 degrees C, and it was made to breathe out by part for 0.8ml/from the inside (0.4mmphi) of an annular spinning port outlet. The distance of about 10mm was led to the water solution of 35% of acetone concentration, and 0.2% of ammonia concentration controlled at 25**0.4 degrees C after air transit, and it was rolled round the rate for 5m/, controlling the filament which carried out the regurgitation so that an average aperture is set to 75nm in an outside solution.
[0017] Then, use 3% of sulfuric-acid solution in the state of immobilization, and it was made to contract and reproduce, and, subsequently rinsed. The vacuum drying of the water in a hollow filament was carried out for the obtained hollow filament at 20 degrees C after the permutation with the methanol. The outer diameter of the hollow filament obtained in this way was 345 micrometers, and thickness was 30 micrometers. When the average aperture was measured, it was 74**4nm. This hollow filament is used and it is 2 0.01m of film surface products with a well-known approach. Filter equipment was assembled so that it might become.
[0018] On the other hand, Escherichia coli was put into the physiological saline solution, and the homogenizer agitated and ground. When this churning liquid was observed with the electron microscope photograph, the 0.15-100-micrometer piece of a cell was observed. 1.0g was dissolved for the gamma globulin desiccation powder of the cow plasma origin in this solution per 1,000ml of this solution. It is the initial pressure of 0.7kg/cm2 with the filter equipment concerned about the obtained protein solution. It filtered. although filtration was continued until the filtrate total amount became 1l., most rises of filtration pressure are accepted -- not having -- the filtration pressure at this time -- 0.7-0.8kg/cm2 it was . When filtrate was observed with the electron microscope, the piece of a cell was not accepted. Moreover, 50ml - 100ml liquid was measured with the spectrophotometer (wavelength of 280nm) after the liquid before filtration, and filtration, and the permeability of protein was measured. A result is shown in Table 1. The permeability of protein was 100% as shown in Table 1.
[0019] In addition, this protein permeability equivalent to protein recovery from the piece mixing solution of a cell is clear.
[0020]
[Comparative Example(s)] Except that filter equipment used the filter for disinfection of 0.22 micrometers of apertures by Millipore Corp. instead of the filter equipment concerned, filtration and measurement were performed by the same condition and approach as an example 1. A result is united and shown in Table 1. Since filtration pressure went up remarkably and the filtration of it became impossible when the amount of filtrate was set to 100ml, filtration was stopped as shown in Table 1.
[0021] As compared with it of an example, it was only only 1 of amount of filtrate of example 1/10 about this amount of filtrate. Moreover, protein permeability was also as low as 90%, and the piece of a cell was observed a little by the electron microscope photograph of filtrate.
[0022]

[Table 1]

	濾液量	濾液中の細胞片の有無	蛋白透過率
実施例	1,000ml	な　し	100%
比較例	100ml	若干有り	90%

[0023]
[Effect of the Invention] According to this invention, separation purification of the purpose protein can be carried out easily with high precision and efficient from a cell mixing solution like a detailed explanation above.

[Translation done.]